Antifungal activity of a Saharan strain of *Actinomadura* sp. ACD1 against toxigenic fungi and other pathogenic microorganisms

*Activité antifongique d'une souche saharienne d'*Actinomadura *sp. ACD1 contre des champignons toxinogènes et autres microorganismes pathogènes*

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KEYWORDS
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Summary A new strain of actinobacteria, designated ACD1, was isolated from a Saharan soil sample in the Hoggar region (Algeria). Morphological study led to this strain being classified as a member of the *Actinomadura* genus. Phylogenetic analysis based on the 16S rRNA gene showed that the strain is closely related to *Actinomadura sediminis* DSM 45500^T (98.5% sequence similarity). Furthermore, strain ACD1 presented a strong activity against mycotoxigenic and phytopathogenic fungi, including *Aspergillus* and *Fusarium* strains, and other pathogenic microorganisms. The kinetics of antimicrobial activity were investigated on ISP-2, Bennett and TSB media. Four solvents (*n*-hexane, dichloromethane, ethyl acetate and *n*-butanol) were used for the extraction of the produced antibiotic. The highest antimicrobial activity was obtained using the butanolic extract from the ISP-2 medium after seven days of fermentation culture. The active antibiotic was purified by reverse-phase HPLC using a C18 column. The UV-visible and mass spectra were determined. The minimum inhibitory concentrations (MIC) of this antibiotic were determined against pathogenic microorganisms.

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MOTS CLÉS
Actinobactérie ;
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Résumé Une nouvelle souche d'actinobactérie, désignée ACD1, a été isolée à partir d'un échantillon de sol provenant de la région du Hoggar (Algérie). L'étude morphologique de cette souche a permis de la rapprocher du genre *Actinomadura*. L'analyse phylogénétique basée sur le gène codant pour l'ARNr 16S a montré que la souche-type la plus proche phylogénétiquement est *Actinomadura sediminis* DSM 45500^T (98,5 % de similarité). De plus, la souche ACD1 a présenté une forte activité contre des champignons phytopathogènes et mycotoxinogènes, y compris les souches des genres *Aspergillus* et *Fusarium* et d'autres microorganismes pathogènes. Les cinétiques de l'activité antimicrobienne sont réalisées sur les milieux ISP-2, Bennett et TSB. Quatre solvants (le *n*-hexane, le dichlorométhane, l'acétate d'éthyle et le *n*-butanol) ont été utilisés pour l'extraction de l'antibiotique produit. La meilleure activité antimicrobienne a été obtenue en utilisant l'extrait butanolique du milieu ISP-2 après sept jours de fermentation. L'antibiotique actif est purifié par HPLC en utilisant une colonne C18. Le spectre UV-visible et le spectre de masse sont déterminés. Les concentrations minimales inhibitrices (CMI) sont réalisées contre des microorganismes pathogènes.

Introduction

Filamentous fungi and yeasts are the causal agents of several of the most serious diseases of humans and plants. Many fungi, especially species from the genera *Aspergillus*, *Fusarium* and *Penicillium* are capable of producing mycotoxins that cause a toxic response when ingested by humans and animals [13]. Among these mycotoxins, ochratoxin A (OTA), known to induce nephropathies and urothelial tumors, is produced by *Aspergillus westerdijkiae*, *Aspergillus carbonarius* and *Aspergillus niger* [1]; aflatoxins, produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are known to cause cancer [16]; and deoxynyvalenol (DON), produced by *Fusarium* spp., is an inhibitor of protein synthesis [31].

The deficiency of antifungal antibiotics and the increased resistance of fungal species to these molecules are real problems for public health. Therefore, many researchers have focused on the isolation of new antifungal compounds. Actinobacteria represent an excellent resource for the discovery of new interesting antibiotics [6]. The genus *Streptomyces* produces about 80% of all the known actinobacteria antibiotics [11]. In recent years, the rate of discovery of new antibiotics from the genus *Streptomyces* has decreased considerably [7]. Thus, many laboratories around the world are focusing their search for new microbial derived antibiotics on non-*Streptomyces* actinobacteria.

The Algerian Saharan soils, exposed to an arid climate, constitute one of the most attractive sources for several rare actinobacteria genera, such as *Actinomadura*, *Actinopolyspora*, *Amycolatopsis*, *Saccharopolyspora* and *Saccharothrix* [32]. In these extreme conditions, the microorganisms developed a specialized metabolism including many interesting antibiotics, such as dithiolopyrrolones [10,21,25] and anthracyclines [39].

In this context, we isolated from a Saharan soil an actinobacterial strain other than the genus *Streptomyces*. The antimicrobial activity of the extract produced by this strain was studied against several toxigenic fungi and against other human pathogenic microorganisms.

Materials and methods

Isolation and features of the actinobacteria strain

The strain ACD1 was isolated from Saharan soil collected in the Hoggar region, Tamanrasset province (southern Algeria). One gram of dry soil was suspended in 9 mL of sterile deionized water. Serially diluted samples were prepared and aliquots (0.1 mL) of each dilution were plated on chitin-vitamin-agar medium, recommended for the isolation of rare actinobacteria [15]. The medium was supplemented with actidione (80 mg/L) to prevent growth of fungi. The plates were incubated at 30 °C for three weeks.

The morphological and cultural characteristics of strain ACD1 were determined on the International *Streptomyces* Project media: yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3) and inorganic salts-starch agar (ISP-4) [35], and also on the Bennett medium [38]. After incubation at 30 °C for 14 days, morphological characteristics were recorded by the naked eye and by using a light microscope (Motic, B1 Series, Hong Kong). The ISCC-NBS color name chart [18] was used to determine the color of the aerial mycelium, the substrate mycelium and diffusible pigments.

For molecular characterization, strain ACD1 was grown in ISP-2 broth, and genomic DNA was extracted with a DNA extraction kit (MasterPure™ Gram Positive DNA Purification Kit, Epicentre® Biotechnologies, Madison). PCR amplification of the 16S rRNA gene sequence of strain ACD1 was performed as described by Rainey et al. [30] by using two universal primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR products were sequenced using the same primers as above and an automated sequencer. The sequence obtained was compared for similarity with the reference species in the public sequence databases and with the EzTaxon-e server [20].

Target microorganisms

Except some bacteria and yeasts pathogenic to humans, most target microorganisms used were filamentous toxigenic fungi. These toxigenic fungi were:

- four OTA producing strains (*A. carbonarius* M333 and Ac2, *A. niger* An1 and *A. westerdijkiae* ATCC 3174);
- three aflatoxin producing strains (*A. flavus* Af3 and E73 and *Aspergillus parasiticus* CBS 100926);
- two DON producing strains (*Fusarium culmorum* Fc1 and *Fusarium graminearum* Fg1);
- one patulin producing strain (*Penicillium expansum* Pe1).

The other pathogenic target microorganisms used were *Umbelopsis ramanniana* NRRL 1829, *Candida albicans* (M3 and IPA200), *Escherichia coli* E52 and *Staphylococcus aureus* MRSA 639c. *Bacillus subtilis* ATCC 6633 was also selected as a target-germ due to its sensitivity to antibiotics secreted by several actinobacteria strains of Saharan origin [2,8,9, 21,39].

Antagonistic properties of strain ACD1

The streak assay method was used for determining the antimicrobial properties of strain ACD1. Actinobacterium strain ACD1 was first cultivated in a straight line on ISP-2 medium for 10 days at 30 °C. After the incubation period, the target microorganisms were seeded in streaks perpendicular to the actinobacterial strain. The antimicrobial activity was determined by measuring of inhibition distance between target microorganisms and the actinobacterial strain after incubation at 30 °C for 24 h for bacteria and yeasts, and 48 h for filamentous fungi.

Kinetics of antibiotic production, growth and pH

To study the evolution of antimicrobial activity, we used three broth culture media: ISP-2, TSB (tryptic soy broth) and Bennett medium (the final pH of the media was adjusted at 7.2). Each 500 mL Erlenmeyer flask contained 100 mL of medium and was inoculated with 3 mL of the actinobacterium culture grown in the same medium on a rotary shaker (250 rpm) for 48 h at 30 °C. The cultures were incubated at the same conditions for 10 days. The aliquots were collected each day by centrifuging (Sigma mini-centrifuges) 4 mL of homogenized culture broth in Eppendorf tubes for 10 min at 16,000 *g*. The centrifugate was used for determining the evolution of biomass and the supernatant was used to determine antimicrobial activity and pH. The antimicrobial activity against *A. carbonarius* M333 and *Bacillus subtilis* ATCC 6633 was regularly recorded each day by the agar diffusion method. Each well of 10 mm in diameter made in the ISP-2 agar plates (12 g/L of agar) was filled with 100 μL of supernatant. Zones of inhibition (diameter, in mm) were recorded after 24 to 48 h of incubation at 30 °C.

Production, extraction and purification of antibiotic

The producing strain ACD1 was cultivated in 500 mL Erlenmeyer flasks that contained 100 mL of liquid ISP-2 medium, and incubated at 30 °C, under constant agitation of 250 rpm. The extraction of the antibiotic was performed on the day of optimal production rate. The ISP-2 culture broth was centrifuged at 8000 *g* for 20 min to remove the mycelium. Each 60 mL of the cell free supernatant was extracted with an equal volume of the following organic solvents: *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The organic extracts were evaporated to dryness under vacuum on a Rotavapor R-205 (Buchi, Switzerland). The resulting dry extracts were dissolved in 1 mL of methanol and tested against *A. carbonarius* M333 and *B. subtilis* ATCC 6633 by the paper disk diffusion method. The disks of 6 mm in diameter received 60 μL of each extract and were placed on the ISP-2 medium (12 g/L agar) inoculated with target microorganism. Inhibition zones were expressed as diameter and measured after incubation at 30 °C for 24 h for *B. subtilis* ATCC 6633 and 48 h for *Aspergillus carbonarius* M333. The rest of the cell free supernatant was extracted with the optimal solvent.

The final purification of antibiotic was carried out by the semi-preparative HPLC JASCO using an interchrom C18 column (250 × 7.8 mm, 15.0 μm, 1.5 mL/min); mobile phase, a continuous linear gradient solvent system from 20 to 100% methanol in water; detector wavelength = 220 nm. All the peaks fractions were collected and tested against *A. carbonarius* M333.

UV-visible and mass spectrum of antibiotic

The UV-visible spectrum of the antibiotic was determined in methanol solution with a SHIMADZU UV1605 spectrophotometer. The mass spectrum was recorded on an LSQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) with nanospray ion electro-spray ionization (ESI) source.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of pure antibiotic were carried out using conventional agar dilution method [27] on the sixteen target microorganisms cited above. These were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for fungi, containing different concentrations of active compound (0.5, 1, 2, 5, 10, 15, 20, 30, 40, 50, 75 and 100 μg/mL). After a growth period at 30 °C for 24 h for bacteria and yeasts and 48 h for filamentous fungi, the plates were examined for growth and the lowest antibiotic concentration that inhibited the growth of each organism was determined. Mueller Hinton and Sabouraud media, without active compound and inoculated with target organisms, were used as control treatments.

Results

Identification of strain ACD1 to the genus level

The strain ACD1 showed good growth on ISP-2 and Bennett media. The aerial mycelium was white to pink and the substrate mycelium was purplish red. A purplish red diffusible pigment was produced. The detailed macromorphological characteristics of the strain are given in Table 1. The strain ACD1 formed extensively branched nonfragmenting and sterile substrate mycelium. The aerial mycelium bore short chains of spores that were straight to flexuous, hooked and irregular spirals (1—2 turns) in all tested media. Endospores, sclerotia, sporangia, synnemata and whirls were not observed.

Through 16S rRNA sequence analysis, an amplified fragment of 1446 bp was obtained and deposited in the GenBank data library. It was assigned the accession number KT259320. This sequence was compared with those of the reference species of bacteria contained in EzTaxon-e server. The similarity levels were 98.5% with *Actinomadura sediminis* DSM 45500^T and 98.3% with *Actinomadura cremea* subsp. *cremea* DSM 43676^T, the most closely related species.

Table 1 Macromorphological characteristics of strain ACD1 on different media after 14 days of incubation.
Caractéristiques macromorphologiques de la souche ACD1 sur plusieurs milieux de culture après 14 jours d'incubation.

Agar medium	Growth	Production and color of aerial mycelium	Color of substrate mycelium	Production and color of diffusible pigment
ISP-2	+++	++ Pinkish white to light pink	Dark purplish red	+++ Dark purplish red
ISP-3	++	+ White to light pink	Dark purplish red	++ Dark purplish red
ISP-4	+	± Whitish	Light purplish red	+ Moderate purplish red
Bennett	+++	± Whitish	Dark purplish red	+++ Dark purplish red

Growth and production of aerial mycelium and diffusible pigment. +++: good; ++: moderate; +: weak; ±: very weak.

Antimicrobial activity of strain ACD1

The antimicrobial activity of the strain ACD1 on ISP2 medium is shown in Table 2. This strain was active against all target microorganisms, and the distance of the inhibitory zone varied from 5 to 25 mm.

Kinetics of antibiotic production, growth and pH

During the time course of fermentation on broth culture media (ISP-2, Bennett and TSB), the antimicrobial activity was evaluated against *A. carbonarius* M333 and *B. subtilis* ATCC 6633 using the agar diffusion method (well technique). The biological activity started after 4 days against *A. carbonarius* M333 and *B. subtilis* ATCC 6633 on ISP-2 and Bennett media. However, no (or very low) activity was observed on TSB medium (data not shown). The maximum antifungal activity was observed after 7 days, while the maximum activity against the bacterium occurred on day 6. The pH varied between 6.1 and 8.4 during the fermentation. The maximum dry mycelia weight (9.4 g/L) was reached after 6 days of fermentation on ISP-2. The antifungal and antibacterial activities were better on ISP-2 medium by comparison to Bennett medium. Thus, ISP-2 medium was selected as the production medium for the antibiotic (Fig. 1).

Table 2 Antimicrobial activity of strain ACD1 by the streak assay method on ISP2 medium.
Activité antimicrobienne de la souche ACD1 sur milieu solide ISP2 par la technique des stries croisées.

Target microorganisms	Inhibition diameter (mm)
Aspergillus carbonarius M333	25
A. carbonarius Ac2	22
A. flavus Af3	5
A. flavus E73	9
A. westerdijkiae ATCC 3174	25
A. parasiticus CBS 100926	9
A. niger An1	15
Fusarium culmorum Fc1	18
F. graminearum Fg1	14
Penicillium expansum Pe1	18
Umbelopsis ramanniana NRRL 1829	11
Candida albicans M3	5
C. albicans IPA200	5
Bacillus subtilis ATCC 6633	20
Escherichia coli E52	8
Staphylococcus aureus MRSA 639c	10

Detection and purification of antibiotic

After 7 days of fermentation on ISP-2 liquid medium, the antimicrobial activity of different organic solvents was evaluated by paper disk method. The higher antimicrobial activity was obtained by the butanolic extract. The diameters of inhibition obtained were 32 mm for *Aspergillus carbonarius* M333 and 24 mm for *Bacillus subtilis* ATCC 6633 (disk diameter included).

The butanolic extract was injected in HPLC by using a reverse-phase column. The HPLC profile obtained showed a peak with antimicrobial activity, which was designated S9 (retention time: 33.7 min, with 86% of methanol in water) (Fig. 2).

UV-visible and mass spectrum of antibiotic S9

The UV-visible spectra of the pure product S9 exhibited a maximum at 255 nm (data not shown). The mass spectrum of the antibiotic S9 was obtained. The negative mode yielded a pseudo-molecular ion $[M - H] = 447$ (Fig. 3). Thus, the molecular weight of the product was $M = 448$.

Minimum inhibitory concentrations

MICs of pure antibiotic S9 are summarized in Table 3. The MIC values were comprised between 2—100 μg/mL for filamentous fungi and 5—75 μg/mL for bacteria. The strains of *Candida albicans* (M3 and IPA200) were resistant. *A. carbonarius* M333 and Ac2, and *A. westerdijkiae* ATCC 3174 were the most sensitive fungi (2—5 μg/mL). *F. culmorum* Fc1, *P. expansum* Pe1, and to a lesser extent, *F. graminearum* Fg1 and *A. niger* An1, were proved to be relatively sensitive (10—15 μg/mL). *B. subtilis* ATCC 6633 was the most sensitive bacterium (5 μg/mL).

Discussion

The strain ACD1 has an aerial mycelium producing short chains of spores that are straight to flexuous, hooked and

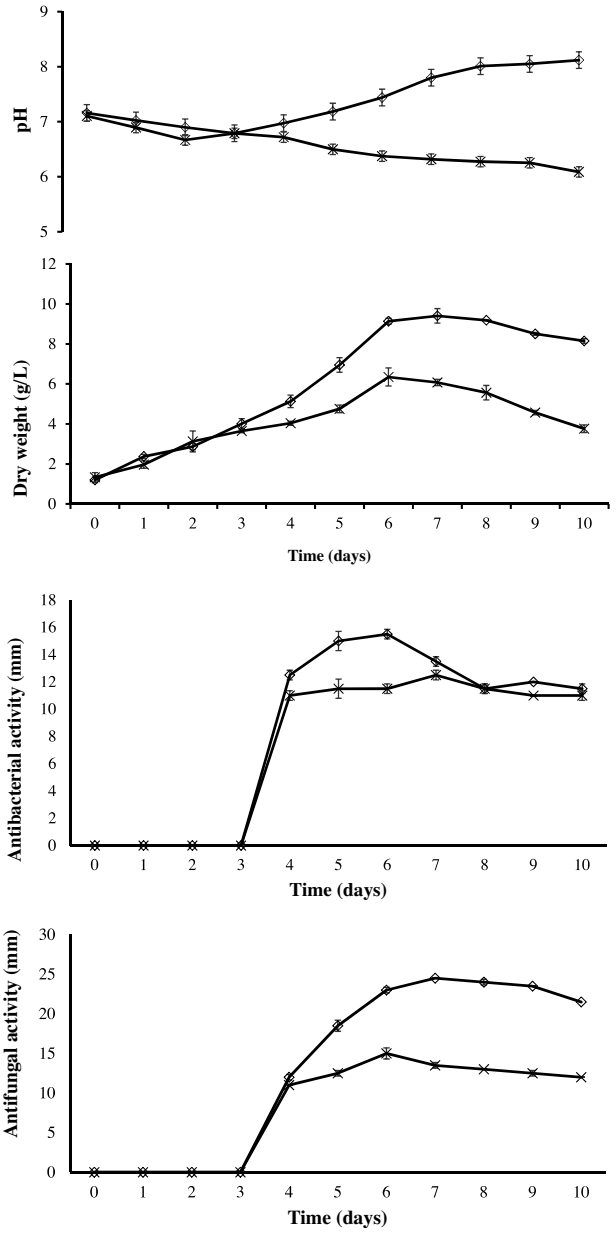

Figure 1 Time course of pH, growth and antimicrobial activity of the strain *Actinomadura* sp. ACD1 on ISP2 and Bennett broth media against *Bacillus subtilis* ATCC 6633 and *Aspergillus carbonarius* M333; ISP2 (◇), Bennett (x).
Cinétiques de l'évolution du pH, de la croissance et de l'activité antimicrobienne de la souche Actinomadura *sp. ACD1 dans les milieux liquides ISP2 et Bennett contre* Bacillus subtilis *ATCC 6633 et* Aspergillus carbonarius *M333 ; ISP2 (◇), Bennett (x).*

spiraled, and a branched nonfragmented substrate mycelium. These morphological characteristics are typical of the genus *Actinomadura* [22]. At the time of writing, the genus *Actinomadura* encompasses 52 species with validly published names (http://www.bacterio.net). The 16S rRNA sequence of strain ACD1 was compared with those of other *Actinomadura* species. The similarity level ranged from 95.1% with *Actinomadura rupiterrae* DSM 45251^T to 98.5% with *Actinomadura sediminis* DSM 45500^T, which is the most closely related species. According to Stackebrandt et al. [36], two microorganisms, which have less than 97% 16S rRNA gene similarity, belong to different species. However, in recent years, other systematists have proposed increasing the threshold for the description of new species. In this context, Meier-Kolthoff et al. [24] suggested 98.2% and Kim et al. [19] proposed 98.65%. Based on the molecular study, strain ACD1 appears to be a new species of *Actinomadura* genus.

Table 3 Minimum inhibitory concentrations (MIC) of the antibiotic S9 produced by the strain ACD1 against several target-microorganisms.
Concentrations minimales inhibitrices (CMI) de l'antibiotique S9 produit par la souche ACD1 contre plusieurs micro-organismes-cibles.

Target-microorganisms	MIC (μg/mL)[a]
Aspergillus carbonarius M333	2
A. carbonarius Ac2	5
A. flavus Af3	100
A. flavus E73	75
A. westerdijkiae ATCC 3174	2
A. parasiticus CBS 100926	75
A. niger An1	15
Fusarium culmorum Fc1	10
F. graminearum Fg1	15
Penicillium expansum Pe1	10
Umbelopsis ramanniana NRRL 1829	30
Candida albicans M3	> 100
C. albicans IPA200	> 100
Bacillus subtilis ATCC 6633	5
Escherichia coli E52	75
Staphylococcus aureus MRSA 639c	40

[a] MIC values represent the average of two repetitions.

Strain ACD1 showed inhibitory activity against pathogenic microorganisms, especially mycotoxigenic and phytopathogenic fungi. Several studies reported the production of various antimicrobial compounds by *Actinomadura* strains, such as kijanimicin [37], decatromicins [26], pradimicin [29] and nomimicin [17]. In Algeria, with the exception of the research carried out by Badji et al. [3—5], little information is available about the antimicrobial activity of *Actinomadura* strains.

The highest activities were observed after 6 to 7 days of fermentation. This means that the strain first grows to form a considerable amount of biomass followed by the production of antibiotic. For actinobacteria, strains with rapid growth produce the antibiotics in the first days of fermentation, such as arylomycin which is produced by a strain of *Streptomyces* after 3 days of incubation [34]. However, other strains belonging to genera with a slow grow rate produce antibiotics during the last days of fermentation, such as pradimicin S produced by a strain of *Actinomadura spinosa* after 12 days of fermentation [33]. In the liquid media tested, antimicrobial activity was observed only on media containing glucose as carbon source, such as ISP-2 and Bennett media, and this is the opposite of what was observed on TSB medium. Glucose can serve as a precursor for antibiotics biosynthesis such as hygromycin A [28] and vancomycin [23]. Among the media, which allowed a good antimicrobial production, ISP-2 medium was found the best

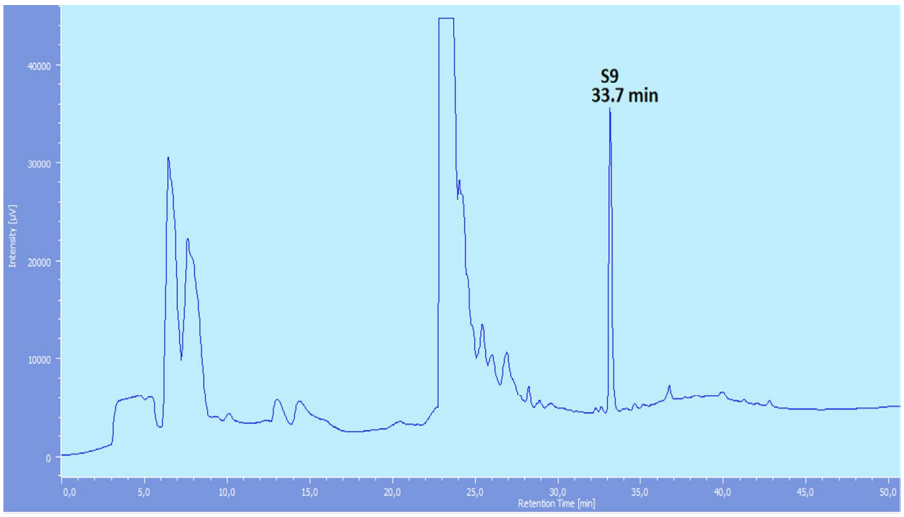

Figure 2 HPLC profile showed the peak corresponding to the active antibiotic S9 secreted by the strain *Actinomadura* sp. ACD1. Column C18 (JASCO); gradient system, 20—100% methanol in water.
Profil HPLC montrant le pic correspondant à l'antibiotique actif S9 secrété par la souche Actinomadura *sp. ACD1. Colonne C18 (JASCO) ; système de gradient, 20—100 % de méthanol dans l'eau.*

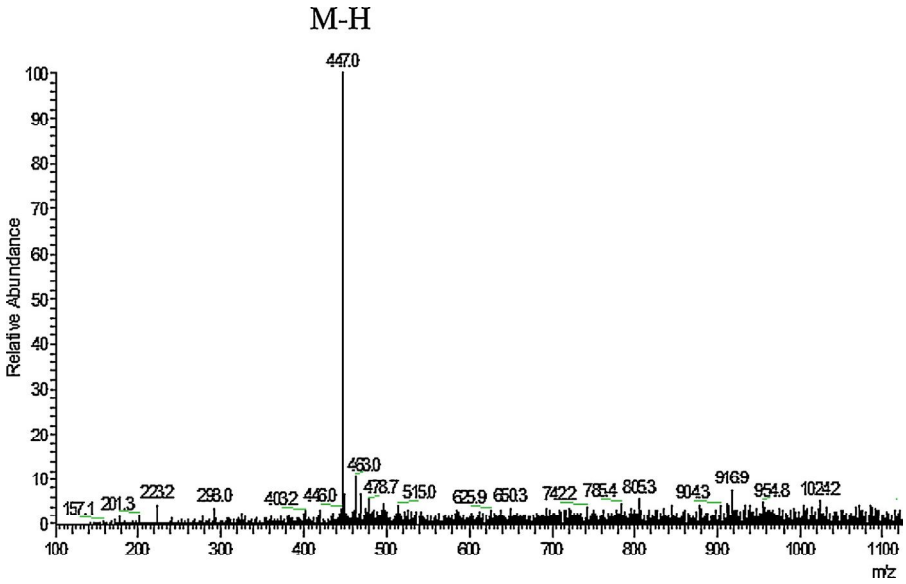

Figure 3 Nanospray ion electron spray ionization-mass spectrum of the antibiotic S9 produced by *Actinomadura* sp. ACD1 in negative mode.
Spectre de masse de l'antibiotique S9 produit par la souche Actinomadura *sp. ACD1.*

for both antibacterial and antifungal activities. This result is supported by several other cases in our laboratory [2,7—9].

The strain of *Actinomadura* sp. ACD1 produced only one antibiotic, named S9 (PM = 448), which has both antibacterial and antifungal activity, and showed a maximum UV absorption at 255 nm. These data indicated that this antibiotic is not polyenic in nature (polyenes are characterized mainly by the antifungal activity and by the three characteristic maxima in the UV-visible). This result is interesting because the polyenic antibiotics are not wanted in the research programs of new antifungal molecules because of many problems related to their toxicity and instability [12,14]. The UV absorption of the antibiotic at 255 nm suggested that this antibiotic contained aromatic moieties. The *Actinomadura* strains, including the isolated strains from Saharan soils, are known to produce the aromatic compounds, as reported by Badji et al. [3—5].

MIC results showed that the activity of antibiotic S9 against filamentous fungi was stronger than the antibacterial one. The same observation was announced by Badji et al. [3,4].

The results obtained in this work are interesting because of the strong activity of antibiotic S9 against mycotoxigenic and phytopathogenic fungi, especially against *A. carbonarius* and *A. westerdijkiae* producing OTA, and also *F. culmorum*, *P. expansum*, and to a lesser extent, *F. graminearum* and *Aspergillus niger*.

Disclosure of interest

The authors declare that they have no competing interest.

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